ATTACHMENT I – PROTOCOL

ECOLAB
Study Identification Number 1000050

REGULATED PESTICIDE EFFICACY STUDY PROTOCOL

STUDY TITLE: DLSB-99 Efficacy at 0.5 oz/gallon to Reduce Foodborne Pathogenic Bacteria in Processing Waters for Fruit and Vegetables

EPA REG. NO.: 1677-

STUDY IDENTIFICATION NUMBER: 1000050

PROPOSED STUDY INITIATION/TERMINATION DATES

Initiation 07/07/10

Termination 08/31/10

DESCRIPTION OF STUDY OBJECTIVE

DLSB-99 (EPA Registration No. **1677-**) will be tested to determine the efficacy as an antimicrobial agent to reduce foodborne pathogenic bacteria in processing waters for fruit and vegetables. Testing will be conducted against three strains of *Listeria monocytogenes* (ATCC 49594, 19114 and 19116), three strains of *Escherichia coli* O157:H7 (ATCC 43895, 35150, 43890) and three strains of *Salmonella enterica* (ATCC 10721, 6962, 13311) after a 5 minute exposure time at 25 ± 2 °C when diluted at 0.5 oz/gallon in 400 ppm synthetic hard water with 1% vegetable soil.

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TEST SUBSTANCE IDENTIFICATION

Test Substance Name: DLSB-99

Batch Identification

1. **P012701**
2. **P032301**
3. **P032401***

All batches aged ≥60 days at time of efficacy testing.
*Batch P032401 will be used for the use-solution chemical analysis.

An aliquot of each batch will be retained in the retention cabinet at ECOLAB Schuman Campus until the quality of the formula no longer affords evaluation. Test or reference substance not dispersed for retention, chemical characterization or efficacy testing will be stored in ECOLAB Microbiological Services cabinet until disposed.

QUALITY ASSURANCE UNIT MONITORING

The protocol, chemical quality verification in-life, chemical quality verification data audit, pesticide efficacy in-life and final report are proposed to be inspected by the ECOLAB Quality Assurance Unit (QAU) in accordance with their current Standard Operating Procedures. The following proposed ECOLAB QA inspections are for planning purposes only and may change. ECOLAB QA inspections that are performed, along with their dates and auditors, will be included in the study final report. Changes in ECOLAB QA inspections from those proposed below will not require revision of this protocol.

A. Proposed QAU Monitoring

Protocol Audit
Chemical Quality Verification In-Life Inspection
Chemical Quality Verification Data Audit
Pesticide Efficacy In-Life Inspection
Final Report Audit

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CHEMICAL QUALITY VERIFICATION

A. Proposed Experimental Initiation/Termination Dates

Initiation 07/14/10

Termination 07/14/10

B. Method

Chemical analysis will be performed on the three batches of test substance to determine the concentration of the active ingredients. Chemical analysis will also be performed on a single batch of test substance use-solution prepared at 0.5 oz/gallon. The use-solution will be prepared by adding 4.30 ± 0.03g test substance to 995.70 ± 0.03g laboratory purified water.

The following calculation was used to determine the amount of test substance in a 1000 g batch at a dilution of 0.5 oz/gallon:

(0.5 oz/gallon) x (1 gallon/128oz) x (Specific Gravity of 1.102 g/mL) x (1000 g)

= 4.30 g test substance

The chemical quality verification will be performed by the Analytical Lab using the methods listed below. The methods listed have been deemed acceptable by the Analytical Lab and the study sponsor to ensure proper characterization of the test substance and test substance use-solution. Statistical treatment of test results may be inherent to the method. Additional volumes and dilutions may be necessary to determine the chemistry of the use-solution samples. Any changes will be noted on the bench sheets.

The most current QATMs and product specific Bill of Quality will be used during the course of this study for the chemical and physical analysis.

Method Number	Method Name
QATM-216A	*Lactic Acid Determination by HPLC*
QATM-279	*Anionic Content by Surfactant Electrode*

QATM-216A; *Lactic Acid Determination by HPLC*
The content of lactic acid is determined by adding excess NaOH and boiling to hydrolyze the anhydride present. Reversed phase liquid chromatography is used to determine the amount of lactic acid with detection by UV absorbance at 210nm and quantitation by comparison of peak area to an external standard.

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QATM-279; *Anionic Content by Surfactant Electrode*
The surfactant electrode responds to the concentration of anionic surfactant in aqueous solution. Using a standardized cationic solution as titrant and the surfactant electrode to identify the endpoint, the concentration of anionic surfactant in an aqueous solution can be determined by titration.

C. Interpretation of Results

The concentration of active ingredients in the test substance will be judged acceptable for pesticide efficacy testing if within the range of 1.17-1.29% LAS (dodecylbenzensulfonic acid, sodium salt) and 16.43-18.15% lactic acid as specified by the Confidential Statement of Formula (CSF) for basic formulation of the test substance. The concentration of the active ingredients in the test substance use-solution will be judged acceptable for pesticide efficacy testing if within the expanded acceptance limits of 0.004766-0.005825% LAS and within the expanded acceptance limits of 0.06699- 0.08188% lactic acid.

The following calculations were used to determine the concentration acceptance criteria for the active ingredients in the test substance use-solution:

Calculated Lower Acceptance Limit = CLAL
Calculated Upper Acceptance Limit = CUAC

CLAL (Active) = CSF LCL x DF
CUAL (Active) = CSF UCL x DF

Dilution Factor = DF = (0.5 oz/gallon) x (1 gallon/128 oz) x (Specific Gravity of 1.102 g/mL) = 0.004305

CLAL (LAS) = 1.17% x 0.004305 = 0.005037%
CUAL (LAS) = 1.29% x 0.004305 = 0.005553%

CLAL (Lactic Acid) = 16.43% x 0.004305 = 0.07073%
CUAL (Lactic Acid) = 18.15% x 0.004305 = 0.07814%

CLAL and CUAL values are equivalent to acceptance criteria for the active ingredients at Nominal (N) ± 5%. After diluting to 0.5oz/gallon, the nominal concentration of the active ingredients are <1.0%. Therefore, CLAL and CUAL will be expanded to accommodate method variability and suitable rationale. The expanded ranges are based on 40CFR158.350 (Certified Limits) and will be calculated as shown below.

Expanded Calculated Lower Acceptance Limit = ECLAL
Expanded Calculated Upper Acceptance Limit = ECUAL

ECLAL (Active) = [N (Active) x DF] – [N (Active) x DF] x 0.1
ECUAL (Active) = [N (Active) x DF] + [N (Active) x DF] x 0.1

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ECLAL (LAS) = [1.23% x 0.004305] – [1.23% x 0.004305] x 0.1 = 0.004766%
ECUAL (LAS) = [1.23% x 0.004305] + [1.23% x 0.004305] x 0.1 = 0.005825%

ECLAL (Lactic Acid) = [17.29% x 0.004305] – [17.29% x 0.004305] x 0.1
= 0.06699%
ECUAL (Lactic Acid) = [17.29% x 0.004305] + [17.29% x 0.004305] x 0.1
= 0.08188%

The chemical quality verification raw data will be reported in the final report of this study.

PESTICIDE EFFICACY TESTING

A. <u>Proposed</u> Experimental Initiation/Termination Dates

Initiation 07/21/10

Termination 07/23/10

B. Methods

Pesticide efficacy data will be generated by the ECOLAB Microbiological Services Laboratory. The SOPs listed below will be used in this study and can be found in the Protocol Appendix. In addition, the Test Method for Efficacy of Antimicrobial Agents to Reduce Foodborne Pathogenic Bacteria in Processing Waters for Fruits and Vegetable will be used in this study and can be found on the next page.

Method Number	Method Name
MS008-21	*Synthetic Hard Water Preparation and Standardization*
MS088-15	*Test Substance Use-Solution Preparation for Analysis*

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Test Method for Efficacy of Antimicrobial Agents to Reduce Foodborne Pathogenic Bacteria in Processing Waters for Fruits and Vegetables

Test System Preparation

A minimum of four consecutive transfers, but less than 15 total transfers, on agar slants need to be made before using to inoculate for testing. Transfers are made on a daily schedule and incubated at 35±2°C. If only one transfer is missed per seven day period, it is not necessary to repeat the three consecutive transfers. If two or more transfers are missed, repeat with three consecutive transfers.

Use the fourth or greater consecutive transfer to inoculate French slants for the test. The transfer used to inoculate the French slants should be 24±4 hours old.

Inoculate French slants by washing the growth from the agar slant into 99 mL of phosphate buffered dilution water (PBDS) as follows:

> Add 5 mL PBDW to the slant and mix to suspend cells. Transfer the cell suspension into the balance of the 99 mL of PBDW.
>
> Mix the suspension well and add 2 mL to each French slant. Tilt the French slant back and forth to cover the agar surface.
>
> Remove the excess suspension aseptically. Incubate French slants in a horizontal position at 35±2°C for 24±4 hours.

Harvest the culture from the agar surface by using 3 mL of PBDW and sterile glass beads and rotating back and forth to suspend the cells.

Collect the cell suspension and beads in a sterile centrifuge tube and vortex approximately 30 seconds to reduce clumping of cells.

Filter the suspension through a sterile Buchner funnel (or equivalent) containing Whatman No. 2 filter paper that has been pre-wet with approximately 1 mL of PBDW. Collect the test system suspension in a sterile test tube and vortex the mixture.

Combine equal volumes of each strain of *Listeria monocytogenes* in a sterile centrifuge tube. Do the same for *Escherichia coli* O157:H7 and *Salmonella enterica*.

Adjust the density of the culture suspension to yield approximately 10×10^{10} organisms per milliliter. This can be accomplished by measuring the % Transmittance (%T) of the culture suspension at 580 nm and adding PBDW as necessary. The % T reading needed to achieve an approximately 10×10^{10} organism/mL culture is suggested as follows: 0.7 %T for *Listeria monocytogenes*, 0.4 %T for *Eschericia coli* O157:H7 and 0.6 %T for *Salmonella enterica*.

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Vegetable Soil Preparation

The test substance will be diluted in 400 ppm synthetic hard water with 1% (vol/vol) sterile vegetable soil added to the synthetic hard water.

The vegetable soil will be prepared as follows:

Measure out 5 grams each of tomato, carrot and Iceberg lettuce.

Blend all produce in a blender with 1000 mL sterile Milli-Q water.

Autoclave the vegetable soil for 13 minutes.

Operating Technique

Dispense 99 mL of the test substance use-solution into a sterile 250 mL Erlenmeyer flask. Prepare triplicate flasks for each test system.

Also prepare triplicate flasks with 99 mL Phosphate Buffered Dilution Water (PBDW) for enumeration of initial numbers control and treat in the same manner as the test flasks.

Place flasks into a 25±2°C temperature water batch and let equilibrate for ≥20 minutes.

Add the test system to the test substance use-solution as follows:

Swirl the test flask, creating enough residual motion to prevent pooling of the test system.

While the liquid is still in motion, place the tip of the pipet containing the test system so it is partially immersed in the test substance use-solution midway between the center and edge of the flask. Add 1 mL of test system suspension to 99 mL of test substance use-solution.

After the exposure period, transfer 1 mL of the test mixture into 9 mL of neutralizer and vortex to mix. This tube is considered a 10^{-1} dilution of the test solution.

Use the pour plate technique to plate in duplicate 1 mL and 0.1 mL of the 10^{-1} dilution from the neutralizer/test solution. In addition, prepare a 10^{-3} dilution in PBDW and plate in duplicate 1 mL and 0.1 mL for the *Listeria* test samples only.

Enumerate the initial numbers control by serial dilution in PBDW. Plate in duplicate using the pour plate technique 1 mL and 0.1 mL from the 10^{-5} dilution and 10^{-7} dilution. The average initial numbers control is valid if it demonstrates a population of $\geq 10^{6}$ CFU/mL

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Controls

Neutralization Controls will be performed as follows:

For each test system, prepare neutralizer control tests A (9 mL neutralizer + 1 mL test substance use-solution), B (9 mL neutralizer + 1 mL PBDW) and C (10 mL PBDW) in triplicate.

Prepare dilutions of each test system suspension in PBDW that result in approximately 10^2, 10^3 and 10^4 CFU/mL.

Add 1 mL of each test system suspension to neutralizer control tests A, B and C and mix.

Plate 1 mL and 0.1 mL from each control (A, B, C) using the pour plate technique.

The neutralization control passes (effectively neutralized the test substance active ingredients) if the average count of control test A and the average count of control test B are with 0.5 $\log_{10}$ CFU/mL of the average count of control test C.

Diluent Sterility Control will be performed as follows:

Plate 1 mL of the diluent using the pour plate technique. The diluent sterility control passes if there is no growth on the diluent sterility agar plate.

Test System Purity Control will be performed as follows:

Inoculate each test system onto Tryptic Soy Agar with 5% Sheep Blood and streak for isolated colonies. Compare the colony morphology and Gram stain result to that typical of the test system.

Incubate plates at 35±2°C for 48±4 hours.

Test Method Requirement and Test System Justification

The test method for use in this study will be Efficacy of Antimicrobial Agents to Reduce Foodborne Pathogenic Bacteria in Processing Waters for Fruit and Vegetables. The test systems which will be utilized for this procedure are three strains of *Listeria monocytogenes* (ATCC 49594, 19114 and 19116), three strains of *Escherichia coli* O157:H7 (ATCC 43895, 35150, 43890) and three strains of *Salmonella enterica* (ATCC 10721, 6962, 13311).

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Test Method Justification

The test method for use in this study will be Efficacy of Antimicrobial Agents to Reduce Foodborne Pathogenic Bacteria in Processing Waters for Fruit and Vegetables.

Test System Identification

The test systems which will be utilized for this procedure are three strains of *Listeria monocytogenes* (ATCC 49594, 19114 and 19116), three strains of *Escherichia coli* O157:H7 (ATCC 43895, 35150, 43890) and three strains of *Salmonella enterica* (ATCC 10721, 6962, 13311). Identification will be performed using the Gram stain technique and by observing the colony morphology on Tryptic Soy Agar with 5% Sheep's Blood.

Statement of Proposed Statistical Method

None

Test Substance Diluent

1% vegetable soil added to 400 ppm Synthetic Hard Water (as $CaCO_3$) prepared as described in ECOLAB Microbiological Services SOP MS008-21; *Synthetic Hard Water Preparation and Standardization* will be the diluent.

Soil Load

1% vegetable soil will be the soil load used in this study since the objective is to determine efficacy in fruit and vegetable processing waters. 1% vegetable soil will be added to 400 ppm Synthetic Hard Water which will be used as the test substance diluent.

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Test Substance Concentration

Determination of the ppm active ingredients in the test substance use-solution if diluted using the test substance with active ingredients at the lower limit at a 0.5 oz/gallon dilution of the test substance:

Resulting ppm of active ingredient = $\left(\frac{\%\ active\ ing.}{100}\right)\left(\frac{\%\ dilution}{100}\right)$ x (SG) (10^6)

Test Substance Active Ingredient	Active Ingredient Lower Certified Limit	Specific Gravity (SG)	Study Proposed Dilution*	Resulting ppm of Active Ingredient
LAS	1.17%	1.102 g/mL	0.3906%	50.4 ppm
Lactic Acid	16.43%			707.2 ppm

*Study Proposed Dilution: (0.5 oz/gallon) (1 gallon/128 oz) (100%) = 0.3906%

Determination of the dilution procedure for each test substance batch will be added by amendment subsequent to the chemical quality verification data generation of the active ingredients in the concentrates. Below are the calculations which will be used to determine the dilution procedure for each batch to ensure both active ingredients are at or below the lower limit.

Determination of the dilution procedure for each batch of test substance to not exceed 50.4 ppm LAS.

Dilution based on % LAS result:

g of the test substance batch in 1000 g to yield 50.4 ppm LAS =

$$\frac{(50.4\ ppm\ LAS)(1000\ g)(100\%)}{(10^6)(\%\ LAS\ in\ batch)}$$

Determination of the dilution procedure for each batch of test substance to not exceed 707.2 ppm lactic acid.

Dilution based on % lactic acid result:

g of the test substance batch in 1000 g to yield 707.2 ppm lactic acid =

$$\frac{(707.2\ ppm\ lactic\ acid)(1000\ g)(100\%)}{(10^6)(\%\ lactic\ acid\ in\ batch)}$$

Exposure Time/Temperature

The test systems will be exposed to the test substance for 5 minutes at 25 ± 2 °C.

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Neutralizer

0.1% Sodium Thiosulfate

Subculture Medium

Brain Heart Infusion Agar (for *Listeria monocytogenes*)
Tryptic Soy Agar (for *Escherichia coli* O157:H7 and *Salmonella enterica*)

Incubation Time/Temperature

The test system plates will be incubated for 48 ± 4 hours at 35 ± 2 °C.

Interpretation of Test Results

The test substance is effective in reducing foodborne pathogenic bacteria in fruit and vegetable processing waters if populations of all test bacteria are reduced by >3 $\log_{10}$ relative to the initial numbers control.

$\log_{10}$ Reduction is calculated as follows:

A - B = $\log_{10}$ Reduction

Where: A = $\log_{10}$ of the average initial numbers control in CFU/mL
B = $\log_{10}$ of the average number of efficacy survivors in CFU/mL

DATA RETENTION

Following completion of the study, an exact copy of the final report and the original raw data and protocol will be transferred to ECOLAB Archives at the ECOLAB Schuman Campus in Eagan, MN or an approved off-site location. All records that would be required to reconstruct the study and demonstrate adherence to the protocol will be maintained for the life of the commercial product plus four years.

TEST SUBSTANCE RETENTION

An aliquot of each batch of test substance will be retained at the ECOLAB Schuman Campus in Eagan, Minnesota until the quality of the formula no longer affords evaluation.

GOOD LABORATORY PRACTICES

This study will be conducted according to Good Laboratory Practices, as stated in 40 CFR Part 160. If it becomes necessary to make changes in the approved protocol, the revisions and reasons for change will be documented, reported to the sponsor and will become part of the permanent file for that study. The sponsor will be notified as soon as it is practical whenever an event occurs that could have an effect on the validity of the study.

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- **Name and Address of Sponsor**

  Sarah Reincke
  ECOLAB
  ECOLAB Schuman Campus
  655 Lone Oak Drive
  Eagan, MN 55121-1560

- **Name and Address of Testing Facility**

  ECOLAB
  ECOLAB Schuman Campus
  655 Lone Oak Drive
  Eagan, MN 55121-1560

- **Name of Study Director**

  Laurinda Holen
  ECOLAB
  ECOLAB Schuman Campus
  655 Lone Oak Drive
  Eagan, MN 55121-1560

Sponsor — Date 7/7/10

Study Director — Date 7/7/10

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PROTOCOL APPENDIX

Microbiological Services (MS) Methods:

MS008-21	*Synthetic Hard Water Preparation and Standardization*	Pages 1-5
MS088-15	*Test Substance Use-Solution Preparation For Analysis*	Pages 1-6

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Standard Operating Procedure
Ecolab, Inc. Controlled Document

2

ECOLAB INC.
MICROBIOLOGICAL SERVICES

TITLE: **Synthetic Hard Water Preparation & Standardization**

NUMBER: **MS008-21**

EFFECTIVE: **02/01/08**

1.0 PURPOSE

To describe how to prepare standardized synthetic hard water solution to be used for diluting products that possess hard water claims.

2.0 SYNTHETIC HARD WATER PREPARATION

2.1 Fill out a media preparation sheet for Solution A and Solution B. Retain in the Media Preparation Log Book.

2.2 Solution A Preparation

Magnesium Chloride ($MgCl_2 \cdot 6H_2O$)	67.74 ± .1 g
Calcium Chloride ($CaCl_2 \cdot 2H_2O$)	97.99 ± .1 g
Sterile Milli-Q Water	1 L

2.2.1 Dissolve powders in 600 mL of boiled Milli-Q water, and then bring to 1 L volume in a 1 L volumetric flask after solution has cooled.

2.2.2 Dispense into appropriate containers (for example, 250 mL Pyrex screw cap bottles) and autoclave for ≥ 15 minutes at ≥ 121°C.

2.2.3 Label using the standard Ecolab labels with a 1 month expiration date and store at 2-8°C.

2.2.4 Quality Control

2.2.4.1 Visual: Clear solution
2.2.4.2 Sterility Check: Sterile after incubation at 32 ± 2°C for ≥ 5 days
2.2.4.3 Expiration Date: 1 month at 2-8°C

2.3 Solution B Preparation

Sodium Bicarbonate ($NaHCO_3$)	56.03 ±.1 g
Sterile Milli-Q Water	1 L

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TITLE: Synthetic Hard Water Preparation & Standardization

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2.3.1 Dissolve in 600 mL of boiled Milli-Q water, then bring to 1 L volume in a 1 L volumetric flask with Milli-Q water after solution has cooled.

2.3.2 Filter sterilize through a 0.45 micron filter into appropriate sterile containers. (approximately 150-200 mL per container)

2.3.3 Label using the standard Ecolab labels with a 1 month expiration date and store at 2-8°C.

2.3.4 Quality Control

2.3.4.1 Visual: Clear solution
2.3.4.2 Sterility Check: Sterile after incubation at 32 ± 2° C for ≥ 5 days
2.3.4.3 Expiration Date: 1 month at 2-8° C

2.4 Hard Water Preparation:

2.4.1 To avoid precipitation of the hard water solution, water should be at room temperature before the addition of Solutions A or Solution B.

Total hardness as ppm $CaCO_3$ = 2.495 x ppm Ca + 4.115 x ppm Mg

2.4.2 To each 1 L of water to be prepared add 1 mL of Solution A for each 100 ppm of $CaCO_3$ hardness desired plus 4 mL of Solution B. (for example, for 500 ppm synthetic hard water add 5 mL of Solution A and 4 mL of Solution B per liter of water)

2.4.3 Bring to 1 L volume with sterile Milli-Q water. If preparing more than 1 L, combine flasks in a sterile 4 L beaker blender after adding appropriate amounts of Solutions A and Solution B and bringing to volume.

2.5 The pH of all test waters less than 2000 ppm hardness (as $CaCO_3$) should be 7.6-8.0. Adjustment of hard water pH using NaOH or HCl may be necessary depending on the starting water pH.

3.0 STANDARDIZATION OF SYNTHETIC HARD WATER

3.1 Method Check - Prior to standardization of the synthetic hard water, the accuracy of the titration method must be checked by analyzing a 500 ppm $CaCO_3$ standard. This must be performed on a monthly basis or when testing new batches of Solutions A and Solution B.

3.1.1 Dilute 10 mL of a 1000 ppm $CaCO_3$ standard (1 mL = 1 mg $CaCO_3$) in 10 mL of Milli-Q water to result in a 500 ppm $CaCO_3$ solution.

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TITLE: Synthetic Hard Water Preparation & Standardization

NUMBER: MS008-21

3.1.2 Dilute 10 mL of the 500 ppm $CaCO_3$ solution in 40 mL of Milli-Q water in a beaker.

3.1.3 Test solution as described in 3.2.2 through 3.2.5.

3.1.4 The hardness of the 500 ppm solution is determined as follows:

hardness (ppm) = (mL EDTA) x 100

3.1.5 Record the result and the lot number of the standard on Form 3011. Hardness of the 500 ppm $CaCO_3$ solution must be 500 ± 20 ppm $CaCO_3$. Failure of the standard to fall within this range indicates a problem in the test method. Corrective actions should be documented in the comments section on Form 3011. The procedure may be used for standardization of synthetic hard water only when results of the standard are within the range described above.

3.1.6 Records from the current and previous year will be kept in the Microbiological Services Equipment Maintenance binder. All earlier records will be archived in the first quarter of the current year. For example, records from 2006 will be archived by March of 2008. Records will be transferred to Ecolab Archives at the Ecolab Schuman Campus in Eagan, MN or to an approved off-site location.

3.2 Sample Testing / Standardization

3.2.1 Dilute 10 mL of prepared hard water in 40 mL of Milli-Q water in a beaker.

3.2.2 Add 1 mL water hardness buffer with magnesium. Use hood when adding; the buffer has irritating vapors.

3.2.2.1 The buffer is VWR product code VW3491 (or equivalent)

3.2.2.2 Approximate composition of buffer, % by weight:

Ammonia	56-57
Ammonium chloride	6-7
EDTA-Magnesium Tetraacetate Salt	0.5
Water	> 35

Note: This buffer has a relatively short expiration.

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TITLE: Synthetic Hard Water Preparation & Standardization

NUMBER: MS008-21

3.2.3 Optional: Add 1mL inhibitor – needed only if previous titration without it has been unsatisfactory. (See 3.2.5.2)

3.2.4 Add just enough Ecolab hardness indicator #016 to yield a pink coloration upon dissolving.

3.2.4.1 Hardness indicator #016 contains Calgamite (1-(1- hydroxy-4-methyl-2-phenylazo)-2-naphthol-4-sulfonic acid) as the actual indicator, along with inert ingredients.

3.2.4.2 It is obtained from Ecolab Test Kits (order through F&B Customer Service) at the Ecolab Engineering Center.

3.2.5 Add 0.01M EDTA slowly until the pink coloration turns blue. Record the number of milliliters of EDTA needed to create the color change.

3.2.5.1 The titration should be completed within 5 minutes of buffer addition to minimize tendency toward $CaCO_3$ precipitation.

3.2.5.2 If the end point color change is not clear and sharp (i.e. the color changes to blue and then drifts back to pink) then an inhibitor / complexing agent must be added (or possibly, the indicator has deteriorated).

3.2.5.3 Prepare inhibitor solution by dissolving 5.0 g sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$) or 3.7 g $Na_2S \cdot 5H_2O$ in 100 mL distilled water. Prepare and dispense in hood. This inhibitor solution deteriorates quickly though air oxidation and should be made each day it is needed.

3.2.5.4 Dilute new sample of test solution and re-titrate beginning with step 3.2.2, including addition of inhibitor.

3.2.6 The hardness of the water is determined as follows:

Hardness as mg $CaCO_3$ / L = (mL EDTA x 1000) / 10 mL of sample
= mL EDTA x 100

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Ecolab, Inc. Controlled Document Standard Operating Procedure

TITLE: Synthetic Hard Water Preparation & Standardization

NUMBER: MS008-21

3.2.7 Upon titration, hardness must not exceed 20 ppm above or below the ppm specified in test procedure / protocol / lab statement. (Therefore, if a claim is for 500 ppm, the titration must yield 500 ± 20 ppm.) If ppm hardness is out of the established range, the sample should be retitrated. Upon a second titration, if ppm hardness is still outside established ranges, the hard water must be diluted or additional solution added to yield the desired ppm. After adjustments have been made, the water must be titrated to determine ppm hardness.

3.2.8 Only two adjustments may be made to the hard water following the above procedure. If the hard water is outside the established limits after two adjustments, the water must be disposed of and the process reinitiated.

3.2.7 For GLP testing, record Hard Water Preparation and Standardization on Form 3010 or Form 3113.

4.0 RELATED FORMS

4.1 Form 3010: Synthetic Hard Water Preparation & Standardization
4.2 Form 3011: Water Hardness Standard Results
4.3 Form 3072: Solution A Prep Log
4.4 Form 3074: Solution B Prep Log
4.5 Form 3113: Test Substance Use-Solution Preparation for Analysis

5.0 REFERENCES

5.1 AOAC (2005) Method 960.09 (E, F)
5.2 APHA, Standard Methods for the Examination of Water & Wastewater, 20th Ed., 1998. Section 2340 C. EDTA Titrimetric Method.

6.0 MOST RECENT REVISION SUMMARY

Minor grammar, spelling and format corrections throughout. Replaced 'the Water Hardness Standard Results Sheet' with 'Form 3011' in 3.1.5. Added the year in 5.1. Added section 6.0.

Prepared by: Linda Briseno Date: 1-9-08
Quality Assurance: Brandy Koslo Date: 1/9/08
Management: Mary C Bruno Date: 1/10/08 7/7/10 EXACT COPY

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Standard Operating Procedure
Ecolab, Inc. Controlled Document

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MICROBIOLOGICAL SERVICES

TITLE: **Test Substance Use-Solution Preparation for Analysis**

NUMBER: **MS088-15**

EFFECTIVE: **07/01/10**

1.0 PURPOSE

To describe the preparation and active ingredient analysis of a diluted test substance (test substance use-solution). Use-solution analysis is included with pesticide efficacy studies, chemical quality verification studies and contract lab studies to verify that the active ingredient concentration corresponds to the dilution made for the claimed active ingredient concentration in the undiluted test substance.

2.0 PROCEDURE

2.1 Typically, use-solutions are prepared as follows:

- 2.1.1 Use-solutions prepared according to the label are for chemical quality verification (CQV) studies
- 2.1.2 Use-solutions prepared at the LCL are for efficacy studies
- 2.1.3 Use-solutions prepared at the UCL are for contract lab TOX studies

2.2 Determine the concentration of active ingredient in the test substance concentrate to verify it is within claimed limits. Perform the analysis for each active ingredient in the product.

2.3 Deionized water may be used as the test substance diluent or the test substance diluent (e.g. hard/soft water or label instructed diluent) may be prepared in the same manner as used for pesticide efficacy testing.

2.4 Prepare the test substance use-solution according to label instructions or as specified in protocol using diluent as described in 2.3. This use-solution should be labeled according to M032.

Example: A 1:64 dilution is 1 part test substance, 63 parts diluent.

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2.5 Analyze the test substance for active ingredient concentration using the same validated QATM that is, or will be, included in the finished good Bill of Quality (BOQ).

Note: The method used to measure active ingredient concentration in the use-solution may have limited sensitivity, accuracy and precision for quantitating the minimal levels of active ingredient found in many use-solutions. These factors may need to be considered when interpreting results. Any modifications to the QATM to adjust for this should be specified in the protocol.

2.6 Analyze the results. The active ingredient concentration in the use-solution should correspond to the dilution made for the claimed active ingredient concentration in the concentrate (e.g. EPA Upper & Lower Certified Limits) and to 40 CFR § 158.350 Certified Limits unless otherwise noted in the protocol. A scientific explanation must accompany any result which does not correspond to the dilution made for the claimed active ingredient level in the concentrate.

3.0 Formulas to Determine Use-solution Amounts and Acceptance Criteria

3.1. Dilution Factor (DF) Determination

3.1.1 Dilution Factor by Volume ($DF_{vol/vol}$)

Example: Dilution Factor ($DF_{vol/vol}$) $= \frac{1\,oz}{1\,gallon} \times \frac{1\,gallon}{128\,oz} = 0.0078$

3.1.2 Density/Specific Gravity (SG) Calculation

Obtain density or specific gravity values from confidential statement of formula (CSF) or suitable documentation. Convert as necessary to g/mL or unitless for SG.

Conversion Example: $\frac{9.2\,lbs}{gallon} \times \frac{1\,gallon}{3785.412\,mL} \times \frac{453.5924\,g}{1\,lb} = 1.102\,g/mL$

$$\text{Density of Product} = \frac{\text{mass (g)}}{\text{volume (mL)}}; \quad \text{Specific Gravity} = \frac{\text{Density of Product}}{\text{Density of Water (1.0 g/mL)}}$$

$$\text{Density of Product} = 9.2\ lbs/gal \sim 1.102\ g/mL; \quad \text{Specific Gravity} = \frac{1.102\,g/mL}{1.0\,g/mL} = 1.102$$

3.1.3 $DF = DF_{vol/vol} \times SG$

$DF = 0.0078 \times 1.102 = 0.0086$

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3.2. Use-solution prepared per label (e.g. 1000 g use-solution prepared at 1 oz/gallon dilution)

3.2.1 Target mass (g) of product = [Total use-solution mass (g)] × DF

Target mass (g) of product = 1000 g x 0.0086 = 8.6 g

3.2.2 Target mass (g) of diluent = [Total use-solution mass (g)] – [Target mass (g) of product]

Target mass (g) of diluent = 1000 g – 8.6 g = 991.4 g

3.2.3 Include a range of ± 0.03 g (~ 1 drop) or ± 0.3 g (~ 10 drops) to target masses when preparing use-solutions.

Note: any appropriate total use-solution mass may be used.

3.3. Use-solution prepared at CSF lower certified limit (LCL) – 1 active ingredient

3.3.1 Determine the active ingredient concentration (ppm) in the test substance use-solution when diluted (per label or protocol) using the test substance (concentrate) with active ingredient(s) at the LCL.

Example: 1 oz/gallon

$$\%\,\text{Dilution} = \frac{1\,\text{oz Product}}{1\,\text{gallon}} \times \frac{1\,\text{gallons}}{128\,\text{oz}} \times 100\% = 0.781\%$$

$$\text{ppm active at LCL} = \frac{\%\,\text{Active at LCL}}{100\%} \times \frac{\%\,\text{Dilution}}{100\%} \times \text{Specific Gravity} \times 10^6$$

$$\text{Target mass (g) of product} = \frac{\text{ppm Active at LCL} \times \text{Total mass of use - solution} \times 100\%}{10^6 \times (\%\,\text{Active Ingredient Result})}$$

3.3.2 Target mass (g) of diluent = [Total use-solution mass (g)] – [Target mass (g) of product]

Note: any appropriate total use-solution mass may be used.

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3.4. Use-solution prepared from CSF lower certified limit (LCL) – multiple active ingredients

- Ensure that all active ingredients are at or below the calculated lower acceptance limit.
- This can be determined by calculating all active ingredient amounts and using an amount (of product) that ensures all active ingredients present to be less than or equal to the calculated lower acceptance limit.

3.4.1 Follow 3.3 to determine target masses (g) of product and diluent.

Note: any appropriate total use-solution mass may be used.

3.5. Use-solution prepared at CSF upper certified limit (UCL) – 1 active ingredient

3.5.1 Follow 3.3 and replace LCL values with UCL values.

Note: any appropriate total use-solution mass may be used.

3.6. Use-solution prepared at CSF upper certified limit (UCL) – multiple active ingredients

- Ensure that all active ingredients are at or above the calculated upper acceptance limit.
- This can be determined by calculating all active ingredient amounts and using an amount that ensures any active ingredient present to be greater than or equal to the calculated upper acceptance limit.

3.6.1 Follow calculations in 3.5 (replace LCL values with UCL values) to determine target masses (g) of product and diluent.

Note: any appropriate total use-solution mass may be used.

3.7. Acceptance criteria formulas and calculations for label dilution use-solutions

3.7.1 **Example:** Product diluted at 1oz/gallon (product/diluent)

Where: CSF UCL = 18.15%; CSF LCL = 16.43%; DF = 0.0086; Nominal (N) = 17.29%

Note: Calculated lower acceptance limit = CLAL
Calculated upper acceptance limit = CUAL

CLAL (Active) = CSF LCL x DF = 16.43% x 0.0086 = 0.141%
CUAL (Active) = CSF UCL x DF = 18.15% x 0.0086 = 0.156%

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CLAL and CUAL values are equivalent to acceptance criteria for the active ingredient at Nominal (N) ± 5% at label dilution (1 oz/gallon). After dilution to 1 oz/gallon the nominal concentration of the active ingredient will be ≤ 1.0%.

When this happens, the calculated upper/lower acceptance limits may be expanded to accommodate method variability or suitable rationale. Expanded ranges are based on 40CFR158.350 (Certified Limits). Include justification in the protocol when expanding acceptance criteria.

If the nominal concentration (N) for the ingredient is:	The certified limits for that ingredient will be as follows	
	Upper Limit	Lower Limit
N ≤ 1.0%	N + 10%	N - 10%
1.0% < N ≤ 20.0%	N + 5%	N - 5%
20.0% < N ≤ 100.0%	N + 3%	N - 3%

Note: Expanded calculated lower acceptance limit = ECLAL
Expanded calculated upper acceptance limit = ECUAL

ECLAL (Active) = [N (Active) × DF] - {[N (Active) × DF] x 0.1}
ECUAL (Active) = [N (Active) × DF] + {[N (Active) × DF] x 0.1}

ECLAL (Active) = [17.29% × 0.0086] - {[17.29% x 0.0086] × 0.1} = 0.134%
ECUAL (Active) = [17.29% × 0.0086] + {[17.29% x 0.0086] × 0.1} = 0.164%

3.7.2 CLAL/CUAL and ECLAL/ECUAL may be labeled as applicable in the protocol by the study director.

3.7.3 Products with a CSF UCL/LCL that is greater than N ± 10; CLAL/CUAL calculations should be determined as in 3.7.1 but acceptance criteria may NOT be expanded.

3.8. Acceptance criteria formulas and calculations for use-solutions diluted to the CSF LCL or UCL.

3.8.1 **Example:** Product diluted to 1 oz/gallon

Acceptance criteria for use-solutions diluted to the CSF LCL or UCL are greater than or equal to the CLAL/CUAL.

Acceptance Criteria (Active at CSF LCL) = CSF LCL × DF = 16.43% × 0.0086 = 0.141%
Acceptance Criteria (Active at CSF UCL) = CSF UCL × DF = 18.15% × 0.0086 = 0.156%

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Therefore: Acceptance Criteria (Active at CSF LCL) ≤ 0.141%
Acceptance Criteria (Active at CSF UCL) ≥ 0.156%

3.0 RELATED FORMS

3.1 Form 3113: Test Substance Use-Solution Preparation for Analysis

4.0 REFERENCES

4.1 M032: Labeling Requirements
4.2 40 CFR 158.350

5.0 MOST RECENT REVISION SUMMARY

Revised section 1.0 for clarity. Added a new 2.1. Added a new section 3.0 for use-solution preparation and acceptance criteria determination.

Prepared by: _____ Date: 6/10/10
Quality Assurance: Sherri St. Clair Date: 6/11/2010
Management: _____ Date: 6/11/10

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GLP Study Protocol Amendment

Study Title:	DLSB-99 Efficacy at 0.5 oz/gallon to Reduce Foodborne Pathogenic Bacteria in Processing Waters for Fruit and Vegetables
Study Number:	1000050
Amendment Number:	1000050-1A
Amendment Effective:	07/20/10

Description of Amendment

1. The protocol is being amended to change the neutralizer from 0.1% Sodium Thiosulfate to D/E Broth.

2. The protocol is being amended to change the volumes to be plated for the neutralization controls. Rather than plating 1 mL and 0.1 mL from each control A, B and C, only 1.0 mL will be plated in duplicate from each control A, B and C.

3. The protocol is being amended to remove the requirement to prepare a 10^{-3} dilution of the neutralizer/test solution and plate 1 mL and 0.1 mL for the *Listeria* test samples. The neutralizer/test solution for each test system will be plated in duplicate by plating 1 mL and 0.1 mL of the 10^{-1} dilution.

4. The protocol is being amended to include a justification for the selection of the test systems. The test systems were chosen since they are pathogenic bacteria found on fruits and vegetables.

5. The protocol is being amended to include the actives in the Study Objective. The test substance will be diluted at or below the lower limit of 50.4 ppm LAS and at or below the lower limit of 707.2 ppm lactic acid.

6. The protocol is being amended to clarify the ECLAL and ECUAL calculations on page 4 and 5 of the protocol. An additional bracket was added to clarify the order of the calculations to be performed as shown below:

ECLAL (Active) = [N (Active) x DF] – {[N (Active) x DF] x 0.1}
ECUAL (Active) = [N (Active) x DF] + {[N (Active) x DF] x 0.1}

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7. The protocol is being amended to include the dilution procedure for each batch of test substance following the chemical quality verification data generation performed on the active ingredients in the concentrates.

Determination of the dilution procedure for each batch of test substance to not exceed 50.4 ppm LAS.

Dilution based on % LAS result:

g of the test substance batch in 1000 g to yield 50.4 ppm LAS =

$$\frac{(50.4 \text{ ppm LAS})(1000 \text{ g})(100\%)}{(10^6)(\% \text{ LAS in batch})}$$

Determination of the dilution procedure for each batch of test substance to not exceed 707.2 ppm lactic acid.

Dilution based on % lactic acid result:

g of the test substance batch in 1000 g to yield 707.2 ppm lactic acid =

$$\frac{(707.2 \text{ ppm lactic acid})(1000 \text{ g})(100\%)}{(10^6)(\% \text{ lactic acid in batch})}$$

To ensure the test substance use-solution batches are at or below 50.4 ppm LAS and 707.2 ppm lactic acid, the test substance use-solution batches will be prepared based on the lactic acid concentration as shown in bold in the following table. An equivalent dilution may be prepared.

Test Date	Test Substance Batch Identification	Active Ingredient	% Active Ingredient in Batch	Amount of Test Substance: Amount of Diluent
07/15/10	P012701	LAS	1.17%	4.31 g : 995.69 g
		Lactic Acid	**17.2%**	**4.11 g : 995.89 g**
	P032301	LAS	1.27%	3.97 g : 996.03 g
		Lactic Acid	**18.1%**	**3.91 g : 996.09 g**
	P032401	LAS	1.20%	4.20 g : 995.80 g
		Lactic Acid	**17.5%**	**4.04 g : 995.96 g**

*Weights may vary +/-0.03g.

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Scientific Basis for Amendment

1. The protocol was amended to change the neutralizer from 0.1% Sodium Thiosulfate to D/E Broth. D/E Broth effectively neutralizes both active ingredients in the test substance.

2. The protocol was amended to change the volumes to be plated for the neutralization controls. Rather than plating 1 mL and 0.1 mL from each control A, B and C, only 1.0 mL will be plated in duplicate from each control A, B and C. It was redundant to plate 1 mL and 0.1 mL from neutralizer controls inoculated with approximately 10^2, 10^3 and 10^4 CFU/mL.

3. The protocol was amended to remove the requirement to prepare a 10^{-3} dilution of the neutralizer/test solution and plate 1 mL and 0.1 mL for the *Listeria* test samples since the preparation of that dilution is not necessary.

4. The protocol was amended to include a justification for the selection of the test systems.

5. The protocol was amended to include the actives in the Study Objective.

6. The protocol was amended to clarify the ECLAL and ECUAL calculations on page 4 and 5 of the protocol. An additional bracket was added to clarify the order of the calculations to be performed.

7. The protocol was amended to include the dilution procedure for each batch of test substance following the chemical quality verification data generation performed on the active ingredients in the concentrates.

☐ This amendment does affect the integrity of the study.

☒ This amendment does not affect the integrity of the study.

Study Sponsor — *Date* 7/23/10

Study Director — *Date* 7/21/10

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GLP Study Protocol Amendment

Study Title:	DLSB-99 Efficacy at 0.5 oz/gallon to Reduce Foodborne Pathogenic Bacteria in Processing Waters for Fruit and Vegetables
Study Number:	1000050
Amendment Number:	1000050-2A
Amendment Effective:	08/03/10

Description of Amendment

The protocol is being amended to allow for the repeat efficacy testing of all 3 batches of DLSB-99 against the three strains of *Salmonella*. When efficacy testing was performed on 07/21/10 and read on 07/23/10, the results obtained were not consistent between replicates nor consistent between batches. In addition, growth was consistent with that confirmed to be a contaminant, *Escherichia coli*. Eventhough efficacy testing passed, it will be repeated to confirm efficaciousness of the test substance.

Scientific Basis for Amendment

The protocol is being amended to allow for the repeat efficacy testing of all 3 batches of DLSB-99 against the three strains of *Salmonella*. When efficacy testing was performed on 07/21/10 and read on 07/23/10, the results obtained were not consistent between replicates nor consistent between batches. In addition, growth was consistent with that confirmed to be a contaminant, *Escherichia coli*. Eventhough efficacy testing passed, it will be repeated to confirm efficaciousness of the test substance.

☐ This amendment does affect the integrity of the study.

☒ This amendment does not affect the integrity of the study.

Study Sponsor — *Date* 8/4/10

Study Director — *Date* 8/3/10

Printed & Verified Initial & Date 8/3/10

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Effective: 06/01/10
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GLP Study Protocol Amendment

Study Title:	DLSB-99 Efficacy at 0.5 oz/gallon to Reduce Foodborne Pathogenic Bacteria in Processing Waters for Fruit and Vegetables
Study Number:	1000050
Amendment Number:	1000050-3A
Amendment Effective:	09/02/10

Description of Amendment

The protocol is being amended to clarify the minimum number of consecutive transfers of the test system that need to be made prior to inoculating the French slants for efficacy testing. Page 6 of the protocol states both four consecutive transfers and three consecutive transfers. The protocol is being amended to clarify a minimum of three consecutive transfers needs to be made prior to inoculating the French slants for efficacy testing.

Scientific Basis for Amendment

The protocol was amended to clarify a minimum of three consecutive transfers of the test system needs to be made prior to inoculating the French slants for efficacy testing. Page 6 of the protocol stated both four consecutive transfers and three consecutive transfers.

☐ This amendment does affect the integrity of the study.

☒ This amendment does not affect the integrity of the study.

Study Sponsor — *Date* 9/2/10

Study Director — *Date* 9/2/10

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Effective: 09/18/09
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GLP Study Protocol Deviation

Study Title:	DLSB-99 Efficacy at 0.5 oz/gallon to Reduce Foodborne Pathogenic Bacteria in Processing Waters for Fruit and Vegetables
Study Number:	1000050
Deviation Number:	1000050-1D
Date Deviation Occurred:	07/13/10

Description of Deviation

For QATM 216A; *Lactic Acid Determination by HPLC*, when the sodium hydroxide solution (R lot 2) was prepared on 07/13/10, it was prepared using approximately 20 g of sodium hydroxide and 500 mL Milli-Q water. Per QATM 216A-006, the sodium hydroxide solution should have been prepared with 40 g of sodium hydroxide and 1000 mL Milli-Q water.

Justification for Deviation

The test method calls for 1N sodium hydroxide. The analyst prepared 1N sodium hydroxide by using an equivalent dilution. The solution prepared using 20 g of sodium hydroxide in 500 mL Milli-Q water has the same normality intended for the boiling solution (1N). The solution prepared using 20 g of sodium hydroxide in 500 mL Milli-Q water is equivalent to the solution prepared per QATM 216A. The use of the equivalent solution did not compromise the data integrity.

☒ This deviation does not affect the integrity of the study.

☐ This deviation does affect the integrity of the study.

Study Sponsor — *Date* 8/25/10

Study Director — *Date* 8/25/10

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